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Lab Resource: Stem Cell Line

Generation of induced pluripotent stem cells (iPSCs) from a retinoblastoma patient carrying a c.2663G>A mutation in RB1 gene

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ABSTRACT

Skin fibroblasts were obtained from a male patient diagnosed with retinoblastoma (RB) carrying a c.2663G>A mutation in the 25 exon of RB1 gene. RB-iPS cells was generated via delivered four reprogramming factors (OCT4, SOX2, NANOG and LIN28) into these skin fibroblasts. The RB-iPS cells retained the RB1 heterozygous mutation resulted in a truncated RB1 mRNA. Characteristic tests proved that the iPSC line presented typical markers of pluripotency and had the capability to form the three germ layers in vitro.

Resource table

Name of stem cell construct	RB-iPS cell line
Institution	National Engineering and Research Center of Human Stem Cell
Person who created resource	Yan Zhao
Contact person and email	Sicong Zeng: sicong520@csu.edu.cn
Date archived/stock date	Mar 20, 2016
Origin	Human skin fibroblasts
Type of resource	Biological reagent: induced pluripotent stem cell (iPS); derived from RB1 p.S888A heterozygous mutation male patient
Sub-type	Induced pluripotent stem cells (iPSCs)
Key transcription factors	OCT4, SOX2, NANOG, LIN28
Authentication	Identity and purity of cell line confirmed (Fig. 1)
Link to related literature (direct URL links and full references)	N/A
Information in public databases	N/A

Resource details

Induced pluripotent stem cells (iPSCs) was derived from a male retinoblastoma patient after informed consent. Sequencing analysis confirmed a heterozygous missense mutation c.2663G>A (p.S888A) in the 25 exon of RB1 in RB-iPS cells (Fig. 1A). This mutation located within exon and intron junction which may cause the alternative splicing. By using primers that spans the junction of exons 23 and 26, two different size products of RB1 gene were found in RB patient-derived skin fibroblast cells (RB-SF) and RB-iPS cells, but not in the normal skin fibroblast cells (N-SF) (Fig. 1B). Similar to the human embryonic stem cell line (chHES-8) which were obtained from the established hESC bank at our center (Lin et al., 2009), the RB-iPS cells showed high telomerase activity compare to the skin fibroblasts and expressed the pluripotency related genes by RT-PCR (Fig. 1C–D). During long-term culture on the mitotically inactivated mouse embryonic fibroblasts (MEFs), the RB-iPS cells maintained a stable karyotype 46, XY (Fig. 1E), and were positive for OCT4, NANOG, SSEA4, TRA-1-60 and TRA-1-81 as well as alkaline phosphatase (Fig. 1F). The differentiation capacity of RB-iPS cells was confirmed through in vitro assays. The cells from embryoid bodies expressed the key genes related with the development of main organs from all three germ layers, such as ectoderm marker β-TUBULIN, mesoderm marker SMA and endoderm marker AFP (Fig. 1G).

Materials and methods

Ethical approval

All procedures described in this work were approved by the ethical committee of Reproductive and Genetic Hospital of CITIC-Xiangya. The skin biopsy used for generation of the induced pluripotent stem cell line were isolated from a RB-diagnosed patient with c.2663G>A mutation in RB1 gene (p.S888A) after written informed consent.

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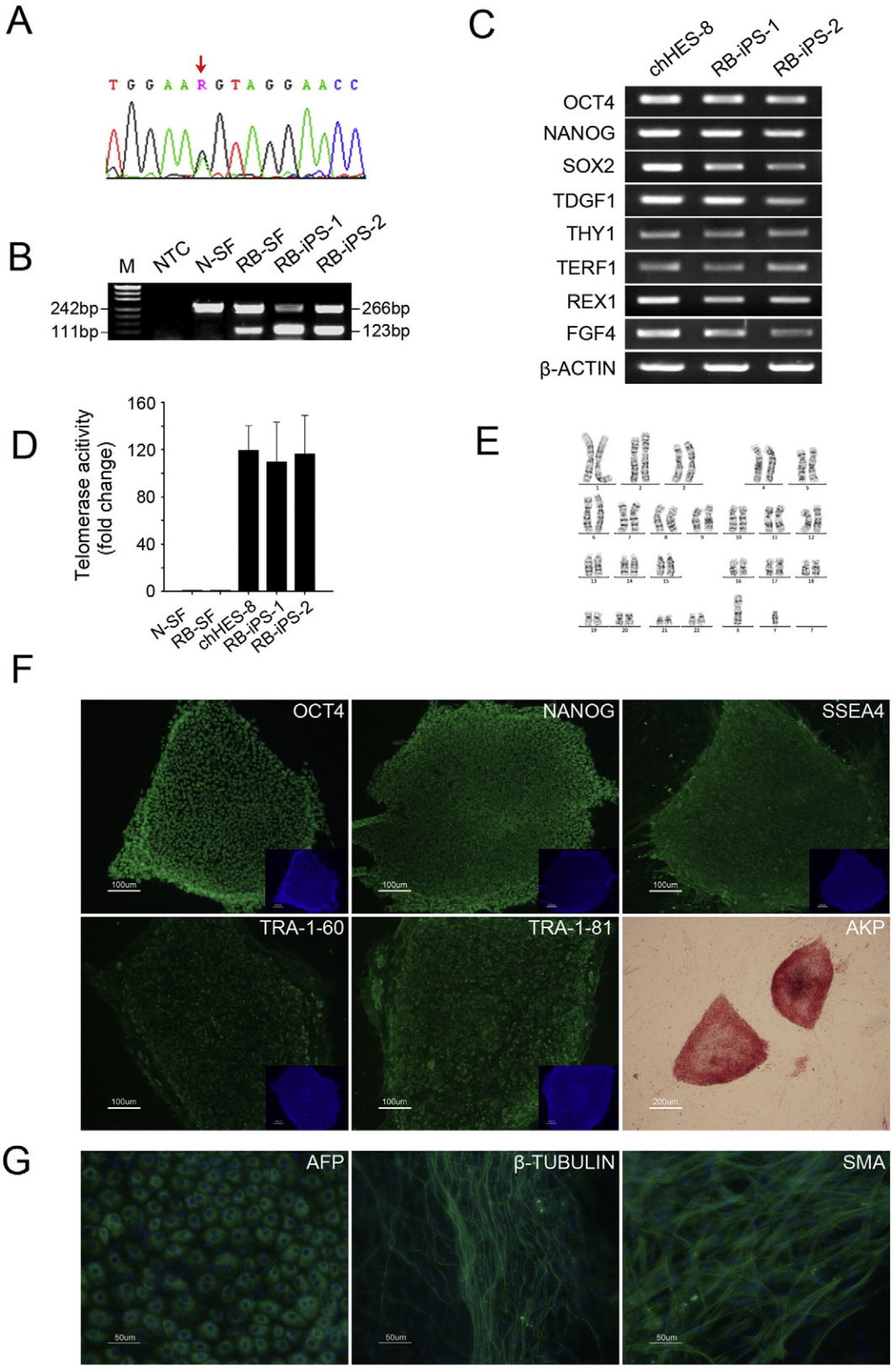

Fig. 1. Characterization of RB-iPS cell line. (A) Sequencing of exon 25 of the RB1 gene in RB-iPS cells showing a heterozygous c.2663G>A substitution (red arrow). (B) Heterozygous deletion of RB1 assessed by RT-PCR. The RB-iPS-1 and RB-iPS-2 cells exhibited two bands of 266 bp and 123 bp, resemble the skin fibroblast from RB patient (RB-SF), indicating the heterozygous mRNA deletion between the exon 23 and the exon 26 of RB1 gene. The normal human skin fibroblast (N-SF) exhibited only one band of 266 bp, indicating no heterozygous mRNA deletion; NTC, non-template control; M, DNA Marker. (C) Gene expression analysis by Semi-quantitative RT-PCR. Pluripotent genes were detected in the undifferentiated RB-iPS cells. (D) Quantitative analysis of telomerase activity in RB-iPS cells, hESCs and SF. (E) RB-iPS cells had a normal karyotype 46, XY. (F) RB-iPS colonies were positive for OCT4, NANOG, SSEA4, TRA-1-60, TRA-1-81 and AKP. Scale bar = 100 μm. (G) Derivatives from embryoid bodies of RB-iPS cells could differentiate into ectoderm (β-TUBULIN), mesoderm (SMA) and endoderm (AFP) in vitro. Scale bar = 50 μm.

Table 1
List of primers used in RT-PCR.

Genes	Primer sequence	Temp. (°C)	Product size (bp)
OCT4	5′-CTTGCTGCAGAAGTGGGTGGAGGAA-3′ 5′-CTGCAGTGTGGGTTTCGGGCA-3′	64	168
NANOG	5′-ACTGTCTCTCCTCTTCCCTCCTCC-3′ 5′-GTAGAGGCTGGGGTAGGTAGGTG-3′	64	387
SOX2	5′-AGTCTCCAAGCGACGAAAAA-3′ 5′-GCAAGAAGCCTCTCCTTGAA-3′	54	142
TDGF1	5′-TCCTTCTACGGACGGAACTG-3′ 5′-AGAAATGCCTGAGGAAAGCA-3′	56	139
THY1	5′-AGAATACCAGCAGTTCACCCATCC-3′ 5′-CCTCACACTTGACCAGTTTGTCTCTG-3′	58	237
TERF1	5′-GCAACAGCGCAGAGGCTATTATT-3′ 5′-AGGGCTGATTCCAAGGGTGTAA-3′	58	159
REX1	5′-TGAAAGCCCACATCCTAACG-3′ 5′-CAAGCTATCCTCCTGCTTTGG-3′	58	556
FGF4	5′-GCGTGGTGAGCATCTTCG-3′ 5′-GGTGACCTTCATGGTGGG-3′	54	230
β-actin	5′-CGCACCACTGGCATTGTCAT-3′ 5′-TTCTCCTGGATGTCACGCAC-3′	60	200

Derivation of the iPS cell line

The skin fibroblasts from the skin biopsy were maintained in Dulbecco's modified eagle medium (DMEM, Hyclone) containing 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine and 1% penicillin and streptomycin. 3×10^5 skin fibroblasts in 60 mm dish were infected twice with four lentiviruses which packaged by using the following vectors: pSin-EF2-OCT4-Pur (Plasmid 16579 Addgene), pSin-EF2-SOX2-Pur (Plasmid 16577 Addgene), pSin-EF2-NANOG-Pur (Plasmid 16578 Addgene), pSin-EF2-LIN28-Pur (Plasmid 16580 Addgene). The pMDG and pCMV△R8.91 were gifted from professor Benjamin Reubinoff (Hadassah University Hospital, Israel). After transduction was completed, the cells were cultured with ES medium comprising of 15% knockout serum replacement, 2 mM L-glutamine, 2 mM nonessential amino acids, 0.1 Mm β-mercaptoethanol and 4 ng/mL of basic Fibroblast Growth Factor (bFGF) (all from Thermo). The medium was changed every day. Fifteen or twenty days after transduction, colonies were picked up and transferred on MEFs. Undifferentiated cell colonies were passaged mechanically per week for long-term culture.

PCR-Sanger sequencing

Genomic DNA was prepared with QIAmp® DNA mini kit (Qiagen). Mutation in the 25 exon of RB1 gene was examined by PCR amplification. Following primers were used for PCR amplification reaction: 5′-AGGTCTGCCAACACCAACAA-3′ and 5′-TTTCAGTGGTTTAGGAGGGT-3′. Cycling conditions were as follows: 95 °C for 5 min followed by 35 cycles of amplification (95 °C denaturation for 30 s, 62 °C annealing for 30 s, 72 °C elongation for 30 s) and a final extension at 72 °C for 5 min. The PCR products were determined by Sanger sequencing using a BigDye Terminator cycle sequencing kit v3.1 and an ABI 3130XL Genetic Analyzer (Applied Biosystems).

Semi-quantitative RT-PCR analysis

Total RNA was prepared using the RNeasy Mini Kit (Qiagen), according to the manufacturer instructions. 1 μg of total RNA was reverse transcribed using the SUPERSCRIPT III Reverse Transcriptase (Thermo) according to the manufacturer's instructions. PCR reactions were performed with GoTaq polymerase (Promega) on 0.2 μL of a reverse-transcription reaction mix in a total reaction volume of 10 μL. PCR products were separated using 2% agarose gels and visualized with ethidium bromide staining. The product sizes, annealing temperatures and primer sequence are listed in Table 1.

Telomerase activity measurement

The telomerase activity of the iPSCs was measured using a TRAPeze RT Telomerase Detection Kit (Millipore S7710) according to the manufacturer's instructions.

Karyotype analysis

iPSCs were cultured on matrigel (Corning) with mTeSR1 (Stem Cell) were treated with KaryoMAX® Colcemid™ solution (Gibco) for 3 h and harvested with Accutase (Millipore), and then used for standard G-banding karyotype analysis.

Immunocytochemistry and alkaline phosphatase staining

The cells were harvested and fixed for 15 min in 4% paraformaldehyde in PBS at room temperature, and then blocked and permeabilized with PBS containing 0.5% Triton X-100 (Sigma) and 10% donkey serum (Jackson Immuno Reserach) for 30 min at room temperature for 30 min. After incubated with the primary antibodies for mouse anti-OCT4 (1:200, Santa Cruz), rabbit anti-NANOG (1:100, Abcam), mouse anti-TRA-1-60 (1:50, Millipore), mouse anti-TRA-1-81 (1:50, Millipore), mouse anti-SSEA4 (1:50, Millipore), mouse anti-β-TUBULIN (1:800, Sigma), mouse anti-AFP (1:500, Sigma), and mouse anti-SMA (1:100, Millipore), at 4 °C overnight. After rinsing to remove the unbound antibodies, samples were exposed to Alexa Fluor® 488 donkey anti-mouse lgG (1:1000, Thermo) for 1 h in the dark. Nuclei were stained with DAPI (Thermo). In addition, alkaline phosphatase activity was detected with BCIP/NBT Kit (Thermo). Images were examined using a Nikon ECLIPSE TE2000-U fluorescence microscope (Nikon, Japan).

In vitro differentiation

iPSCs were mechanically dissociated into small clumps and cultured as aggregates in suspension for 7 days to form embryoid bodies (EBs) in ES medium without bFGF. The EBs were then adherently cultured on gelatin-coated 6-well for 14 days. Differentiated cells were fixed and stained immunostained with three

germ layer marker (endoderm: AFP; ectoderm: β-TUBULIN; mesoderm: SMA).

Verification and authentication

Karyotyping and Sequencing analysis were performed at Reproductive & Genetic Hospital of CITIC-Xiangya. All 20 metaphase cells observed had the normal 46, XY karyotype, free of any discernible abnormalities. PCR-Sanger sequencing confirmed a heterozygous missense mutation c.2663G>A (p.S888A) of RB1 in RB-iPS cells which is consistent with that of the proband.

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